

# EXHIBIT 9

## Gene Amplification and Drug Resistance in Cultured Murine Cells

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In this article we review studies in our laboratory concerning the molecular mechanism for the development of resistance of cultured mouse cells to the 4-amino analog of folic acid methotrexate (MTX). We show that this resistance results from a selection of cells with higher contents of a specific enzyme, dihydrofolate reductase (DHFR) and corresponding increases in the number of cop-

ylizes the reduction of dihydrofolate to tetrahydrofolate (3). Tetrahydrofolate is required for single carbon transfer (such as  $-\text{CH}_3$  or  $-\text{CH}_2^-$ ) reactions for glycine, purine, and thymidylate synthesis. Thus inhibition of DHFR prevents de novo synthesis of key precursors of proteins and nucleic acids.

Continued administration of MTX to patients often results in the emergence of

**Summary.** Resistance of mouse cells to the folate analog, methotrexate, results from selection of increasingly resistant cells on progressive increases of methotrexate in the culture medium. High-level resistance is associated with high rates of synthesis of dihydrofolate reductase and correspondingly high numbers of reductase genes. In some variants high resistance and gene copy number are stable in the absence of selection pressure, whereas in others they are unstable. Analogies are made to antibiotic and insecticide resistance wherein selection of organisms with increased capacity to counteract the drug effect results in emergence of resistance. Gene amplification may underlie many such resistance phenomena.

ies of the gene coding for this enzyme, that is, gene amplification. In some cells the amplified genes are stable in the absence of selection pressure (MTX), whereas in others the genes are unstable. The properties of this resistance are analogous to many cases of the emergence of drug resistance in bacteria and insects (1). Thus, gene amplification may be also involved in these instances.

### Methotrexate Resistance in Cultured Cells

Methotrexate, a 4-amino analog of folic acid, is commonly used in the treatment of malignancy (2). It kills cells by specifically inhibiting dihydrofolate reductase (DHFR), the enzyme that cata-

is not inhibited (10). Resistance by mechanisms (ii) and (iii) rarely occurs spontaneously but can be enhanced by mutagenesis (11). Cultured cells can also become resistant to MTX by combinations of the above mechanisms.

### Molecular Mechanism for Increased Dihydrofolate Reductase

We have been studying the molecular mechanism responsible for the increased content of DHFR in resistant cells derived from the murine sarcoma cell line S-180 (6). Resistant cells (AT-3000 and clones derived from that cell line) are resistant to 3000 times the MTX concentration that kills sensitive cells (S3) from which the resistant line was derived. The AT-3000 cells contain approximately 200 times as much DHFR as the S3 cells, as judged by enzyme assays and stoichiometric binding of MTX to DHFR (6) as well as by immunologic criteria in which a highly specific antibody is used (12).

Experiments on the labeling of cellular protein with radioactive amino acids and isolating DHFR by specific antibody precipitation indicate that the 200-fold increase in enzyme can be accounted for entirely by an increased rate of its synthesis (12). A similar conclusion has been made by Hanggi and Littlefield for a MTX-resistant cell line derived from baby hamster kidney (BHK) cells (13). The only protein that is detectably different in extracts of sensitive and of resistant cells is a peak corresponding to a molecular weight of 21,000, the size of the DHFR molecule (Fig. 1). In the resistant cells, DHFR constitutes approximately 3 to 4 percent of the total cell protein. Thus, by this admittedly imprecise criterion, the alteration in resistant cells appears to be limited to the synthesis of a single protein.

In order to quantify the cell content of DHFR specific messenger RNA (mRNA) and the number of DHFR genes, we have used nucleic acid hybridization techniques. The molecular probe used is DNA complementary (cDNA) to dihydrofolate reductase mRNA (14). Figure 2 shows the results of kinetic hybridizations of the cDNA with (i) an excess of total mRNA to quantitate the relative content of DHFR mRNA and (ii) an excess of cellular DNA to determine the relative number of DHFR genes in sensitive and resistant cells. The degree of acceleration of hybridization of cDNA measures the relative differences in the mRNA contents and the number of gene copies. There is an approximately 200-fold acceleration in the rate of hybridiza-

drug-resistant tumors; these tumors are generally associated with an increased content of DHFR (2). Methotrexate resistance can also be obtained in cultured murine and hamster cells. Three mechanisms for this resistance have been described: (i) An increase in DHFR occurs (4-7); highly resistant variants are obtained only by a stepwise selection with progressive increments in MTX in the medium. Treatment of cells with common mutagens does not increase the frequency of emergency of resistance as a result of elevation in DHFR. Resistance accompanying high DHFR levels results from the fact that at any concentration of MTX in the medium, if there is more enzyme, some enzyme molecules will be in an uninhibited state and hence will permit cell growth. (ii) Alterations in the structure of DHFR such that the high affinity for MTX is lost; hence enzyme is no longer effectively inhibited (8, 9). (iii) Alterations in transport of MTX into cells such that the intracellular concentration of MTX is minimal; hence DHFR

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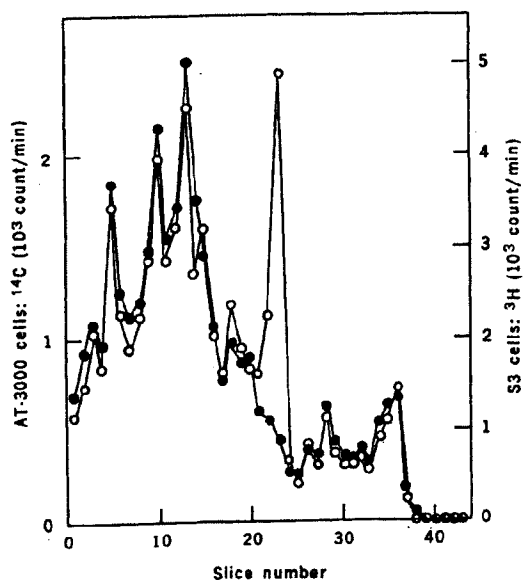


Fig. 1. Comparison of pulse-labeled soluble proteins from sensitive and resistant S-180 cells. Growing cultures of S3 and AT-3000 cells were labeled with [ $^3\text{H}$ ]leucine (●—●) or [ $^{14}\text{C}$ ]leucine (○—○), respectively. Soluble protein extracts were subsequently mixed and subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gels (12). [Courtesy of the *Journal of Biological Chemistry*]

tion of cDNA with RNA from resistant cells as compared to the rate for sensitive cells. Thus, the difference in enzyme content and rate of its synthesis results from a comparable increase in the amount of DHFR mRNA. There is a comparable acceleration in the rate of hybridization of cDNA with DNA, indicating that there is a comparable amplification of the number of DHFR genes.

Table 1 summarizes data for two murine cell lines, indicating the correspondence between enzyme levels, mRNA content, and gene copy number. More recently we have found in other MTX-resistant cell lines with increased DHFR levels, including murine L 5178Y, hamster BHK, and hamster CHO, an increased gene copy number commensurate with the increased enzyme levels (15).

#### Stability and Instability of Cell Resistance to Methotrexate

The AT-3000 line, as well as a number of cloned sublines, were found originally to retain high enzyme levels only when maintained continually in the presence of MTX (12). Instability in the absence of selection pressure has been reported with a number (4, 6, 7) but not all (7, 11) resistant cell lines. High enzyme levels and rates of enzyme synthesis decline rapidly when a clone (R1) of the AT-3000 cells that contain 100 times as much DHFR as S3 cells is grown in the absence of MTX. After approximately 20 cell doublings, the resulting population

of cells contains only 50 percent of the initial enzyme activity (Fig. 3). The same R1 cells that were cultured in 50  $\mu\text{M}$  MTX continuously for 2 years were ex-

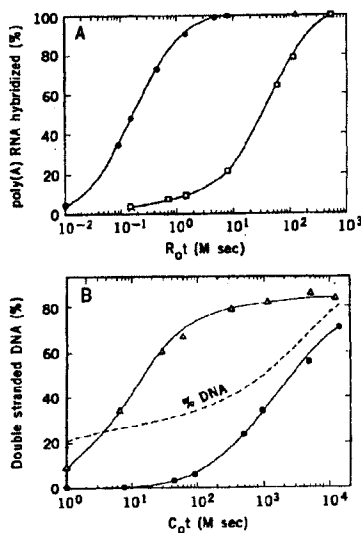


Fig. 2. Comparisons of the relative amount of dihydrofolate reductase RNA and gene number. (A) Hybridizations of tritiated DNA complementary to DHFR mRNA were undertaken with poly(A) RNA from MTX-sensitive (□) cells and MTX-resistant (●) cells. (B) Same as (A) except that DNA was used instead of poly(A) RNA. The dotted line indicates the percent of double-stranded DNA determined from absorbance; ○, sensitive cells; △, resistant cells. The difference in the rate of hybridization is an indication of the relative differences between the two cell populations (12). [Courtesy of the *Journal of Biological Chemistry*]

amined for the rate of reversion of resistance in the absence of MTX (Fig. 3). After 2 years of continuous culture in MTX, the cells reverted to lower-level resistance more slowly and became stably resistant, with enzyme levels approximately 50 times those of sensitive cells. This difference appears to result from changes associated with growth of the cells continually in MTX over a 2-year period, since R1 cells that had been frozen away at the time of the original experiment showed the same rapid reversion to low-level resistance observed originally. Thus, a cell population that was originally unstable with respect to MTX resistance has subsequently become stabilized.

The question arises as to whether the revertant cell population at the point where enzyme levels are 50 percent of the initial levels consists of 50 percent of cells synthesizing DHFR at the high rate and 50 percent at the rate of sensitive cells, or whether it consists of a population that synthesizes DHFR at an intermediate rate. We have studied this question in two ways. We have determined (Fig. 4) the resistance of the cells to MTX (7). When R1 cells were grown in the absence of MTX for more than 400 cell doublings (designated R1A-400), they contained ten times as much enzyme as S3 cells, and they were correspondingly more resistant than the S3 cells to MTX. The R1 clone of AT-3000 was highly resistant, and a 50:50 proportion of R1 and R1A-400 cells showed the expected mixed sensitivity. The R1 cell population (circles of Fig. 3) that has reverted to a point where the synthesis of DHFR was only at 50 percent that of the R1 cells grown in MTX displays a killing curve of cells with approximately 50 percent of the DHFR content of the R1 cells. However, the MTX killing curve of the partially reverted R1 cells is not that of a unique cell population in which each cell contains a uniform content of DHFR. This is in keeping with the heterogeneity in enzyme content and cell (see below). We have also used a method that allows for quantifying DHFR in individual cells (see below) and have come to the same conclusion—namely, that, in the partially reverted population, cells contain approximately 50 percent of the enzyme level of the R1 cells.

#### Quantifying Dihydrofolate Reductase in Individual Cells

Since the cell populations that we study are not likely to be uniform with respect to enzyme content (and gene

copy number), we have devised a method for ascertaining the amount of DHFR in individual cells (16). This method is based on the ability of a fluorescein derivative of MTX to bind specifically, and with high affinity, to DHFR. The quantitative binding allows for the use of the Fluorescent-Activated Cell Sorter both to quantify the enzyme per cell and to separate cell populations on the basis of differing enzyme content. The distribution of cells is plotted as a function of fluorescence per cell (Fig. 5). We have found that fluorescence is a linear function of the specific enzyme activity and the rate of enzyme synthesis (16). In addition we have found that fluorescence is linearly related to gene copy number (15). The S3 cells (Fig. 5C) constitute a uniform population with virtually no fluorescence, whereas the R1A-400 cells constitute a separate and nonoverlapping population with higher enzyme content. Figure 5A shows the fluorescence distribution of the R1 cells grown for 2 years continuously in MTX (see Fig. 4) and shows a skewed distribution at the high fluorescence end (note change in scale). The highly fluorescent cells have been sorted under sterile conditions to obtain cells with high fluorescence (see arrow) and analyzed immediately for their fluorescence distribution (dotted lines). When these cells are grown for ten cell doublings in the absence of MTX, the enzyme levels are not noticeably changed. However, after 7 weeks of growth in the

Table 1. Dihydrofolate reductase, mRNA, and gene copy number in various murine cell lines. The cell lines are described in the text (13). The values are relative.

Cell line	Specific enzyme activity	mRNA sequences	Gene copies
S-180			
S3	1	1	1
AT-3000	250	250	250
L1210			
S	1		1
R	35		35

absence of MTX, enzyme levels are reduced by approximately 40 percent and the fluorescence distribution (Fig. 5B) is correspondingly altered as two discrete cell populations begin to appear; these populations are stable with respect to enzyme levels and MTX resistance in that they show approximately 20 and 50 times as much enzyme as the S3 cells.

By means of these techniques, we have been able to demonstrate a heterogeneity in DHFR levels in cells in which the amplified genes were unstable. When these cells were grown without selection pressure, several different cell populations emerged with differing enzyme content per cell. Inasmuch as the cells sorted for high enzyme content did not overlap with the distribution of cells that eventually stabilized with respect to enzyme content, it is unlikely that our original sorting contained a few cells that had

less enzyme and became the dominant cells upon subsequent growth. We have also cloned a number of cells from the sorted population with very high enzyme content (Fig. 5). Each of these individual clones likewise reverted progressively when grown in the absence of MTX, strengthening our conclusion that individual cells lose capacity for high enzyme synthesis. Reversion with respect to enzyme content is also associated with comparable decreases in resistance to MTX (15).

#### Possible Mechanisms of Gene Amplification and Loss

The following is an interpretation of our results in the context of current thoughts on how gene duplication-amplification events normally occur in organisms. The generation of increasingly resistant cells by stepwise selection with increasing concentrations of MTX suggests that the process resulting in a high degree of gene amplification occurs by steps, beginning with an initial duplication, then selection of those cells with the duplicated DNA sequence, and finally further amplification and further selection.

Gene duplications have been demonstrated in various organisms, including *Escherichia coli*, bacteriophage lambda, *Salmonella typhimurium*, and *Drosophila melanogaster* (17, 18). A high selection

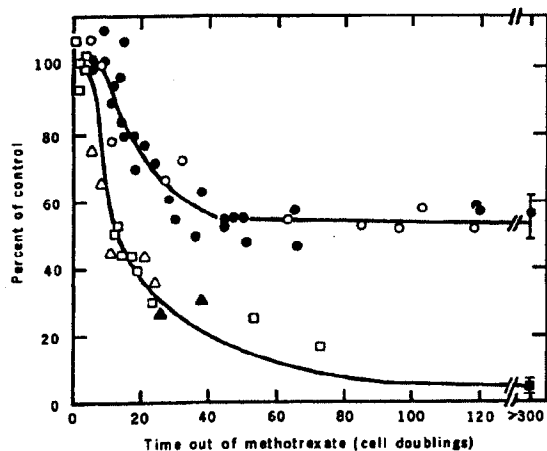


Fig. 3. Loss of dihydrofolate reductase activities in S-180 cells grown in the absence of methotrexate. R1 cells (a clone of AT-3000) were grown in the absence of MTX for various cell doublings. Enzyme activities or rates of DHFR synthesis were determined (15). Squares are cells studied 2 years ago; ■, enzyme levels; □, rates of synthesis. Triangles are those same cells studied in the last 6 months but stored frozen in the interim; ▲, enzyme levels; △, rates of synthesis. Circles are R1 cells grown continuously in MTX for more than 2 years prior to removal from MTX; ●, enzyme levels; ○, rates of synthesis. [Courtesy of the Cold Spring Harbor Symposium on Quantitative Biology]

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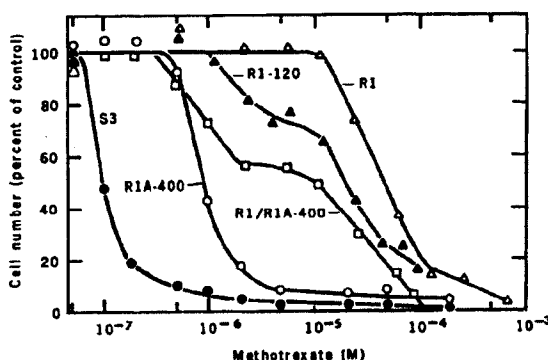


Fig. 4. Methotrexate sensitivity of sensitive, resistant, and revertant cell lines. Methotrexate sensitivity was determined by a growth assay in which  $10^5$  cells were inoculated into media containing different concentrations of methotrexate. When the exponentially growing cultures reached late log phase, the cells were washed, scraped, and counted (Coulter counter). The number of each concentration of methotrexate was calculated from duplicate flasks all of which varied by less than 10 percent. The results were then expressed as a percent of control, which was determined from the growth rate of that line in the absence of methotrexate. The cloned sensitive cell line S3 had a doubling time of 16 hours, whereas all other lines had doubling times of 23 hours. △—△, the resistant R1 line; ●—●, the sensitive line; ○—○ and ▲—▲, low-level revertants and high-level revertants; □—□, a 50:50 mixture of low-level revertants and R1 resistant cells. [Courtesy of Cold Spring Harbor Press]

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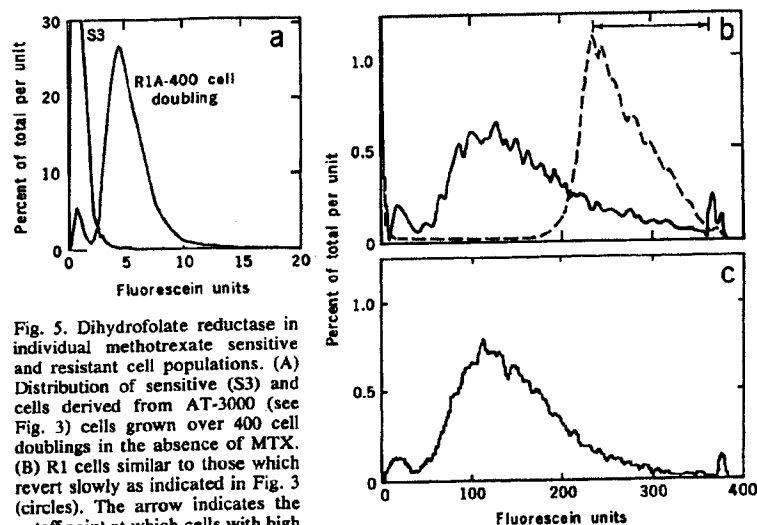


Fig. 5. Dihydrofolate reductase in individual methotrexate sensitive and resistant cell populations. (A) Distribution of sensitive (S3) and cells derived from AT-3000 (see Fig. 3) cells grown over 400 cell doublings in the absence of MTX. (B) R1 cells similar to those which revert slowly as indicated in Fig. 3 (circles). The arrow indicates the cutoff point at which cells with high fluorescence were sorted. The dotted line indicates the fluorescence distribution as analyzed immediately after the sort. (C) The sorted cells when grown 120 cell doublings in the absence of MTX.

pressure was exerted to demonstrate their existence. Anderson *et al.* (18) have estimated that in *S. typhimurium* duplications of genes in the histidine operon occur with a frequency as high as 0.1 percent of the cell population. Interestingly, such duplicated genes can be highly unstable (18) and therefore would not be detected by standard genetic analyses.

We propose that, in the cultured cells that we have been studying, a random duplication of genes occurs as an infrequent event. When grown in low concentrations of MTX, those occasional cells with the duplicated gene will have a growth advantage. An initial DHFR gene duplication could occur by a number of different mechanisms, including unequal crossing over (19) or uptake of a DNA segment from killed cells (20) which is integrated into a chromosome adjacent to the resident gene (21) or disproportionate replication (22).

Once a gene is initially duplicated tandemly, further amplification can occur by unequal crossing over, uptake of DNA from lysed cells, or generation of extrachromosomal sequences from rolling circle replication (23). Various combinations of these mechanisms could result in different genomic organizations for the amplified genes. Cytological studies of Biedler (24) indicate specific abnormalities which were described as "expanded homogeneously staining chromosomal regions" in single chromosomes in MTX-resistant hamster lung cell lines, which are not present in the

sensitive parental cells. We have recently shown that a similar homogeneously staining region on a single chromosome of a MTX-resistant Chinese hamster ovary cell line contains the DHFR genes by in situ hybridization with mouse DHFR cDNA (25). Thus at least in the Chinese hamster ovary cell line, the genes, which are stably amplified, are located to a chromosome.

The loss of genes from cells can occur by the reversal of the processes producing amplification. Unequal crossing over would result in one daughter cell with more genes and one with less. Excision of chromosomal genes with subsequent loss would result in all cells having decreased gene numbers. Thus, a population of cells generated from any given cell with amplified genes would vary with respect to gene number, thereby resulting in a heterogeneous cell population, as is indicated in Fig. 5. When a population of cells is placed under more stringent selection, that is, increased MTX concentration, those cells with more highly amplified genes will be selected. We have found that within a population of cells unstably amplified for DHFR genes, cells with a lower gene copy number have a slightly shorter generation time (15) than those with high gene copy numbers. Hence, when cells are grown without MTX, the population of cells ultimately emerging will appear to have lost genes because of differential growth rates.

Why is it, then, that resistant S-180 cells grown in the presence of MTX con-

tinuously for 2 years have acquired the property of stability with respect to high enzyme content and resistance (and presumably high gene number)? We propose that fixation of stable resistance can be explained on the basis of selection of that population of cells that has maximal growth potential at a fixed MTX concentration. If cells are both amplifying and losing genes continuously, cells that lose too many genes (and lose high DHFR levels) will be killed (Fig. 4) and those cells with highly amplified DHFR genes (relative to that required for growth at the MTX concentration) will be at a growth disadvantage. Consequently, for maximal growth of the cell population, those occasional cells that have a number of genes appropriate for survival at the MTX concentration and that do not undergo loss or amplification will become dominant within the population after a number of generations.

At present we have no definitive information concerning the molecular mechanism (or mechanisms) for fixation of the DHFR genes. Possible mechanisms include (i) translocation of genes from a tandem array such that they cannot undergo recombination events resulting in gene loss; (ii) loss (deletion) of a protein or enzyme involved in recombination processes (19); (iii) integration of unstable, extrachromosomal genes into a chromosomal site (or sites); and (iv) loss of sequences that flank the DHFR genes and are involved in recombination processes. Such proposed sequences would be analogous to "insertion sequences" found in bacterial genomes (26).

Is the amplification of genes in cultured mammalian cells unique to DHFR, or do comparable phenomena occur with other genes? A number of investigators have described the selection of cultured cells resistant to normally lethal concentrations of 25-hydroxycholesterol, deoxynucleosides, and *N*-(phosphoacetyl)-L-aspartate (PALA), which have been found to contain from 2- to 100-fold increases in activities of hydroxymethylglutaryl CoA reductase (E.C. 4.1.3.4) (27), ribonucleotide reductase (E.C. 1.17.4.1) (28), and aspartate transcarbamylase (E.C. 2.1.3.2) (29), respectively.

Stark and his colleagues have recently found that PALA resistance is associated with an amplification of aspartate transcarbamylase genes in their cultured cells (30). Whether other instances of resistance in cultured mammalian cells results from gene amplification awaits further study.

The extent to which amplification of genes underlies other instances of drug



resistance is still unknown. However, the properties of resistance of cultured cells to MTX, including (i) a stepwise selection of progressively resistant cells; (ii) an increase in a specific protein present at low levels in sensitive cells, which, when present in larger amounts, results in resistance; and (iii) stable or unstable resistance in the absence of selection pressure, have analogies both in antibiotic (31) and insecticide resistance (32). Recently Normark *et al.* (33) have, in fact, shown that penicillin resistance in *E. coli* K<sub>12</sub> obtained by stepwise selection results in chromosomal amplification of the gene for  $\beta$ -lactamase (penicillinase).

Our studies with MTX resistance provide further rationale for the principles of drug therapy (whether for bacteria, malignancies, or insects), including the use of multiple drugs, each in sufficient amounts to effect killing separately; treatment for only as long as necessary and with drugs not retained in the environment; and use of a second set of multiple drugs if resistance develops (1, 34). On the basis of the concept of gene amplification as a mechanism of drug resistance, the drugs used should not be counteracted by amplification of a single DNA sequence. Our results suggest that the prolonged administration of a single drug in ever increasing concentrations, which is retained in the environment, is precisely that form of administration

most likely to result in amplification of genes in a stable state, thereby imparting stable resistance.

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## A Proposal to Modernize the American Antiquities Act

Robert Bruce Collins and Dee F. Green

The Act for Preservation of American Antiquities became law in June 1906 (1). The act was passed during a time in U.S. history when people first began to realize that the American frontier, celebrated in Frederick Jackson Turner's epochal paper (2), was not endless, and that the time had come to conserve the nation's natural resources and preserve its historical and archaeological heritage. Since the 1890's there had been great public interest in the art and history of the Indians of the southwestern United

States, and this interest had created a great demand for authentic prehistoric artifacts. As a result, ruins and cliff dwellings, such as Casa Grande, Mesa Verde, and Chaco Canyon, were indiscriminately excavated and vandalized. There were no state and federal laws that provided for the protection of prehistoric sites, and there were few professional archaeologists. Thus, the need for protective legislation was particularly acute when the Antiquities Act was passed in 1906.

The act, which was codified in section 433, title 16 of the U.S. Code, prohibited the appropriating, excavating, injuring, or destroying of any "historic or prehistoric ruin or monument" or "object of antiquity" found on government-owned or -controlled land, without the permission of the secretary of the department of the government having jurisdiction over the land (3). The act was drafted and presented first to the American Anthropological Association and the Archaeological Institute of America by the archaeologist Edgar Lee Hewett. Hewett's draft bill was introduced in the House of Representatives and the Senate in early 1906, and after passage it was signed into law by President Theodore Roosevelt.

The legislative history of the Antiquities Act—that is, the record of debates and reports on the bill in committees and

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